Spermatozoan ultrastructure and detection of nuclear acid phosphatase activity in spermatids of *Anomalocardia brasiliana* and *Tivela mactroides* (Bivalvia: Veneridae)

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ABSTRACT

We compared the ultrastructure of spermatozoa from the bivalves *Anomalocardia brasiliana* and *Tivela mactroides* (Veneridae). The spermatozoa of both species were of the *ect-aquasperm* type in which the head contains a curved nucleus with a short cone-shaped acrosome. An invagination penetrated almost the entire length of the acrosome. The midpiece contained a pair of orthogonally arranged centrioles surrounded by spherical mitochondria and the flagellum had the typical 9 + 2 structure. The spermatozoa of *A. brasiliana* had a slightly curved nucleus while those of *T. mactroides* had a long, prominently curved nucleus. The mitochondria were equally distributed around the centrioles in the midpiece of *A. brasiliana* spermatozoa, but asymmetrically in the midpiece of *T. mactroides* spermatozoa. There were six mitochondria and glycogen clusters in the middle piece of the *T. mactroides* spermatozoon. The presence of glycogen clusters and the higher number of mitochondria, in comparison with *Anomalocardia brasiliana*, could extend the longevity of the *Tivela mactroides* spermatozoa. An increase in sperm life expectancy implies in an increase in the probability of finding eggs and accomplishing fertilization. The glycogen clusters and the higher mitochondria number possibly correspond to an adaptive advantage to the bivalves in turbulent waters.

INTRODUCTION

Comparative studies of the Bivalvia have confirmed the usefulness of spermatozoan morphology for taxonomic and phylogenetic analyses (Bernard and Hodgson, 1985; Guerra et al., 1994; Sousa and Oliveira, 1994; Healy, 1995a, b; Garrido and Gallardo, 1996; Komaru and Konishi, 1996; Healy et al., 2001; Erkan and Sousa, 2002; Gwo et al., 2002; Introíni et al., 2004; Healy et al., 2006). In addition, these studies showed that closely related species can be distinguished based on the ultrastructure of their spermatozoa (Hodgson et al., 1990; Gwo et al., 2002; Introíni et al., 2004).

Various features of spermatozoan ultrastructure have been associated to aspects of reproductive biology. Franzén (1955, 1956, 1977, 1983) proposed that invertebrates have two types of sperm, namely, *primitive* sperm, produced by species with external fertilization, and *modified* sperm, produced by species with internal fertilization. Primitive sperm consists of a short, round or conical head, a midpiece containing 4–5 spherical mitochondria and a flagellum with a 9+2 microtubular structure. Spermatozoa that are released directly into the surrounding water are named *aquasperm* or *aquatic sperm*. Rouse and Jamieson (1987) introduced a new terminology and described two *aquasperm* categories: (1) *ect-aquasperm* referring to sperm that fertilizes eggs in the ambient water; (2) *ent-aquasperm* referring to sperm that fertilizes eggs into the mantle cavity of molluscs or into the tube of sedentary polychaetes.

Sperm morphology has also been correlated with egg size and larval development (Franzén, 1983; Komaru and Konishi, 1996). These relationships among spermatozoan morphology and distinct reproductive patterns have strengthened the relevance of studies that investigate

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the association of evolution, ecology, and the morphological diversity of spermatozoa.

The Veneroida is considered one of the most important orders of bivalves because it comprises several marine families of economic, ecological relevance and widespread geographic distribution, such as the Veneridae (Gwo et al., 2002). Data describing spermatozoan ultrastructure of Veneridae species have supported the identification of traits shared by the majority of the members belonging to this family (Pochon-Masson and Gharagozlou, 1970; Gharagozlou and Pochon-Masson, 1971; Nicotra and Zappata, 1991; Reunov and Hodgson, 1994; Guerra et al., 1994; Matos et al., 1997; Gwo et al., 2002; Erkan and Sousa, 2002; Park et al., 2002; Guerra et al., 2003; Ying et al., 2008).

Numerous reports have emphasized that venerid bivalves are important components of the marine benthos, including non-consolidated bottom communities (Narchi, 1972; Etchevers, 1976; Schaeffer-Novelli, 1980; Prieto, 1980; Soares et al., 1982; Prieto, 1983; Mclachlan et al., 1996; Arruda and Amaral, 2003; Arruda et al., 2003). *Anomalocardia brasiliana* (Gmelin, 1791) is distributed throughout the Caribbean islands and also in Suriname, Brazil and Uruguay (Amaral et al., 2005). This species lives buried a few centimeters below the surface of compact sand in the intertidal zone of calm waters. Adults of *A. brasiliana* rapidly bury themselves when placed on wet mud or muddy sand. *Tivela mactroides* (Born, 1778) occurs around the Ascension Island, along the Caribbean seaboard, and in Venezuela, Suriname and Brazil (Amaral et al., 2005). In contrast to *A. brasiliana*, *T. mactroides* lives in turbulent waters in the intertidal zone.

Although, the spermatozoa ultrastructure of bivalves has been largely investigated and used to solve many taxonomic and phylogenetic issues, immune and cytochemical studies of sperm and precursory cells are scant. Biochemical features of reproductive cell lineages could contribute to taxonomical descriptions and to distinguish some of the bivalve species.

In this work, scanning and transmission electron microscopy were used in the study of the spermatozoan morphology of the venerids *Anomalocardia brasiliana* and *Tivela mactroides*, which were compared with those of other bivalve species, aiming at a better understanding of their taxonomic placement and phylogeny.

MATERIALS AND METHODS

Specimens of *Anomalocardia brasiliana* were sampled in the intertidal zone of São Sebastião County (23°48′57.2″ S, 45°24′29.8″ W), and *Tivela mactroides* sampled in Caraguatatuba County (23°38′51.7″, 45°25′31.4″ W), both located on the southeastern coast of São Paulo State, Brazil.

The voucher specimens were deposited in the Museu de Zoologia "Professor Adão José Cardoso" (ZUEC) at the State University of Campinas (UNICAMP), São Paulo, Brazil, under the accession numbers 1419 (Tivela mactroides) and 1420 (*Anomalocardia brasiliana*).

Scanning Electron Microscopy: Sperm suspensions on coverslips were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.2 M sodium cacodylate, pH 7.2, for 1 h at room temperature. Subsequently, they were rinsed several times in the same buffer and post-fixed in 2% osmium tetroxide in the dark for 1 h. Samples were dehydrated in a graded series of ethanol solutions and critical-point dried in CO2. The dried coverslips were mounted on stubs, coated with gold and examined with a JSM 5800 LV microscope.

Transmission Electron Microscopy: Small fragments of testis were fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.2 M sodium cacodylate, pH 7.2, for 5 h at 4°C and then rinsed in the same buffer. Samples were post-fixed in 2% osmium tetroxide in the same buffer, for 1 h at 4°C, and then dehydrated in a graded acetone series followed by gradual infiltration with EPON resin before embedding. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss Leo 906 transmission electron microscope.

Phosphotungstic Acid Staining: Small samples of testis were fixed as described above, but without post-fixation, and subsequently dehydrated in a graded ethanol series. Samples were stained with a 2% phosphotungstic acid-ethanol (E-PTA) solution at low pH, in order to detect glycoproteins and enable cytochemical analysis of spermatozoa. After 2 h in the E-PTA solution, samples were rinsed with ethanol, transferred to acetone, and infiltrated with EPON resin before embedding. Ultrathin sections were examined with a Zeiss Leo 906 transmission electron microscope.

Nuclear ACPase Detection During Spermiogenesis: Small fragments of testis were fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4, for 2 h at 4°C, and rinsed in the same buffer. They were then washed with 50mM Na-acetate-HCl buffer, pH 5, at 4 °C. Subsequently, the testis fragments were incubated in 50mM Na-acetate-HCl buffer, pH 5, with 5% sucrose, 13.9 mM sodium β-glycerophosphate (β-GP) and 3.6 mM lead nitrate, for 30 min at 37°C, under dark conditions with constant and gentle mixing. Samples were washed twice in 50mM Na-acetate-HCl buffer, pH 5, with 5% sucrose, for 5 min at 4°C, and twice in 0.1 M Na-cacodylate buffer, pH 7.2, with 5% sucrose, for 5 min at 4°C. The specimens were post-fixed in 2% osmium tetroxide in the same buffer, for 2 h at 4°C, and then dehydrated in a graded ethanol series and propylene oxide. A gradual infiltration with EPON resin was done before embedding. Ultrathin sections were examined with a JEOL 100 CX II-TEM, without staining.

The experimental controls consisted of: (1) Omission of β-glycerophosphate; (2) Addition of inhibitor 10mM NaF; (3) Other substrates: 6.4 mM Thiamine pyro-

phosphate chloride; 2.5 mM Na-inosine- 5-diphosphate; 0.6 mM Na-trimetaphosphate.

RESULTS

Spermatozoa: The spermatozoa of both species were either of the *ect-aquasperm* type (Figures 3, 4, and 12). The short acrosomal complex was cone-shaped and located anterior to the nucleus. Two components of the acrosomal vesicle were distinguished based on their diverse electron densities (Figures 20 and 22). The conical acrosome was deeply invaginated, the subacrosomal region was filled with a diffuse material, and there was no axial rod (Figures 1, 2, 10, 11, and 13).

PTA staining at low pH revealed no glycoproteins in the acrosomal vesicles of *Anomalocardia brasiliana* and *Tivela mactroides* spermatozoa (Figures 5, 6 and 14). The nucleus was relatively long, curved, cylinder, and the midpieec consisted of spherical mitochondria grouped around a pair of short cylindrical centrioles (Figures 7, 8, 15, 16, and 17). Extensive electron-dense granules or granule clusters, considered to be glycogen deposits, were observed around the centrioles and mitochondria of *T. mactroides* spermatozoa (Figures 15 and 16). In the region immediately posterior to the midpiece, the triplet substructure of the centrioles was replaced by a standard 9 + 2 microtubular pattern axoneme that terminated in a long flagellum (Figures 9 and 18). Overall, the spermatozoa of *A. brasiliana* and *T. mactroides* shared high morphological similarities, even though the nucleus was slightly curved in the *A. brasiliana* spermatozoa compared to the markedly curved and long nucleus of *T. mactroides* (Table 1, Figures 19 and 24). The mitochondria were equally distributed around the centrioles in the midpiece of *A. brasiliana* spermatozoa (Figure 21). In *Tivela mactroides* spermatozoa, the mitochondrial assembly did not form a ring structure but showed a biased distribution of the organelles around the orthogonally arranged pair of centrioles, such that they were always more numerous in one side of the sperm cell (Figure 23). Hence, the midpiece of *T. mactroides* spermatozoa was rotationally asymmetrical and contained clusters of glycogen, which were not seen in the midpiece of *A. brasiliana*.

Precursory Cells: The nomenclature used to refer to precursory cells was that of Nicotra and Zapata (1991), which is not in accordance with the definitions proposed by Ying et al. (2008).

The early spermatids were rounded but irregular in outline and had a spherical nucleus with patches of condensed chromatin in the middle and in the cell periphery (Figures 25 and 29). The nucleus was still rounded in the mid-spermatid stage, but the chromatin condensation was intensified (Figures 26 and 30).

The late spermatid was characterized by the elongation of the nucleus. Chromatin condensation has completed and only a few nuclear vacuoles remained. While the residual cytoplasm was progressively eliminated, spherical mitochondria assembled in the base of the nucleus around the two centrioles (Figures 27 and 31).

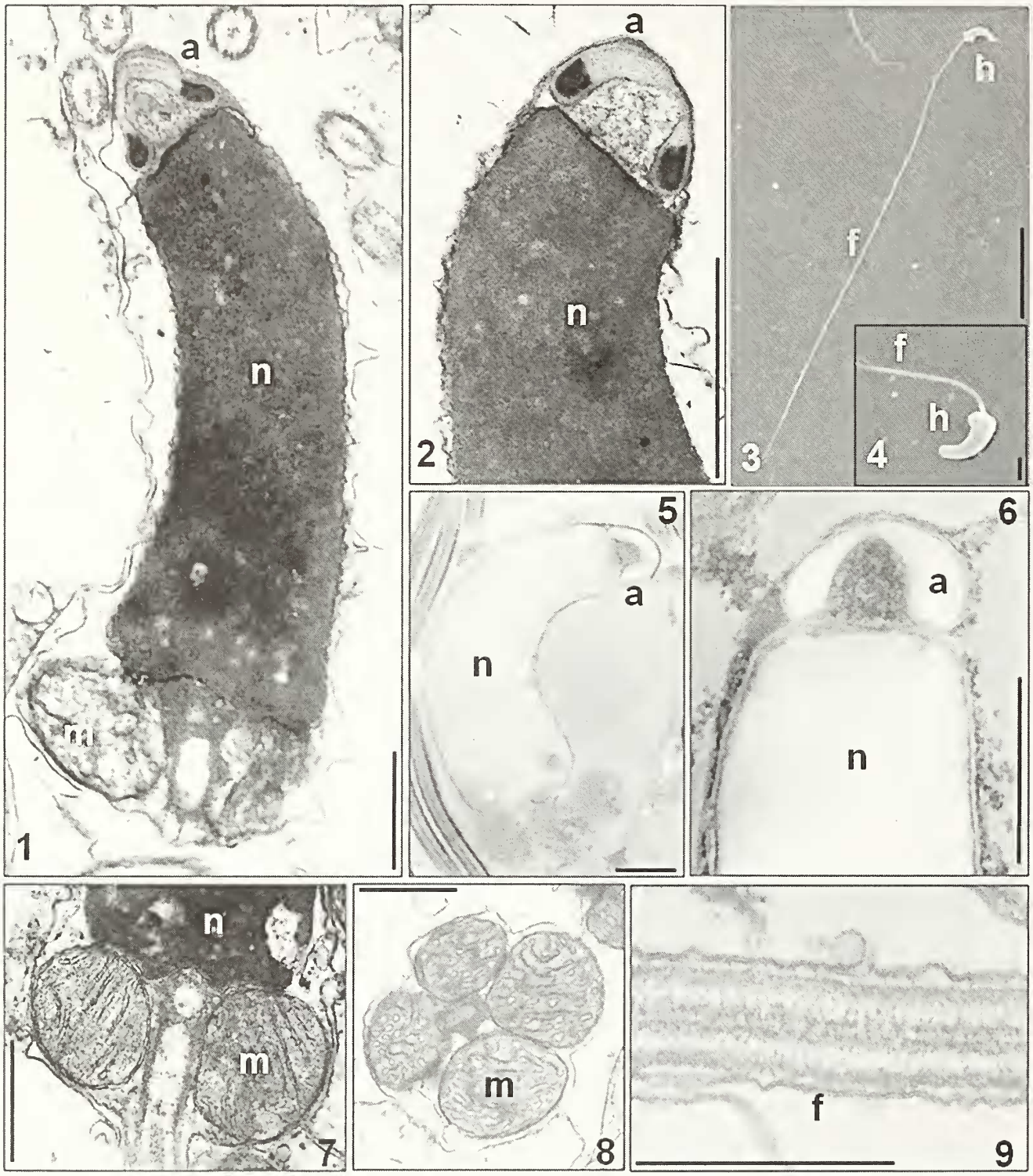
In *Anomalocardia brasiliana* gonads, a nuclear acid phosphatase (ACPase) was detected in all spermatid stages using the improved Gomori-chloride technique. In comparison, in *Tivela mactroides* gonads, the presence of ACPase was detected in mid and (inconspicuously) late spermatids using the same methodology. In the sperm cell of both species, nuclear ACPase was not detected (Figures 28 and 32). The experimental controls did not show staining.

DISCUSSION

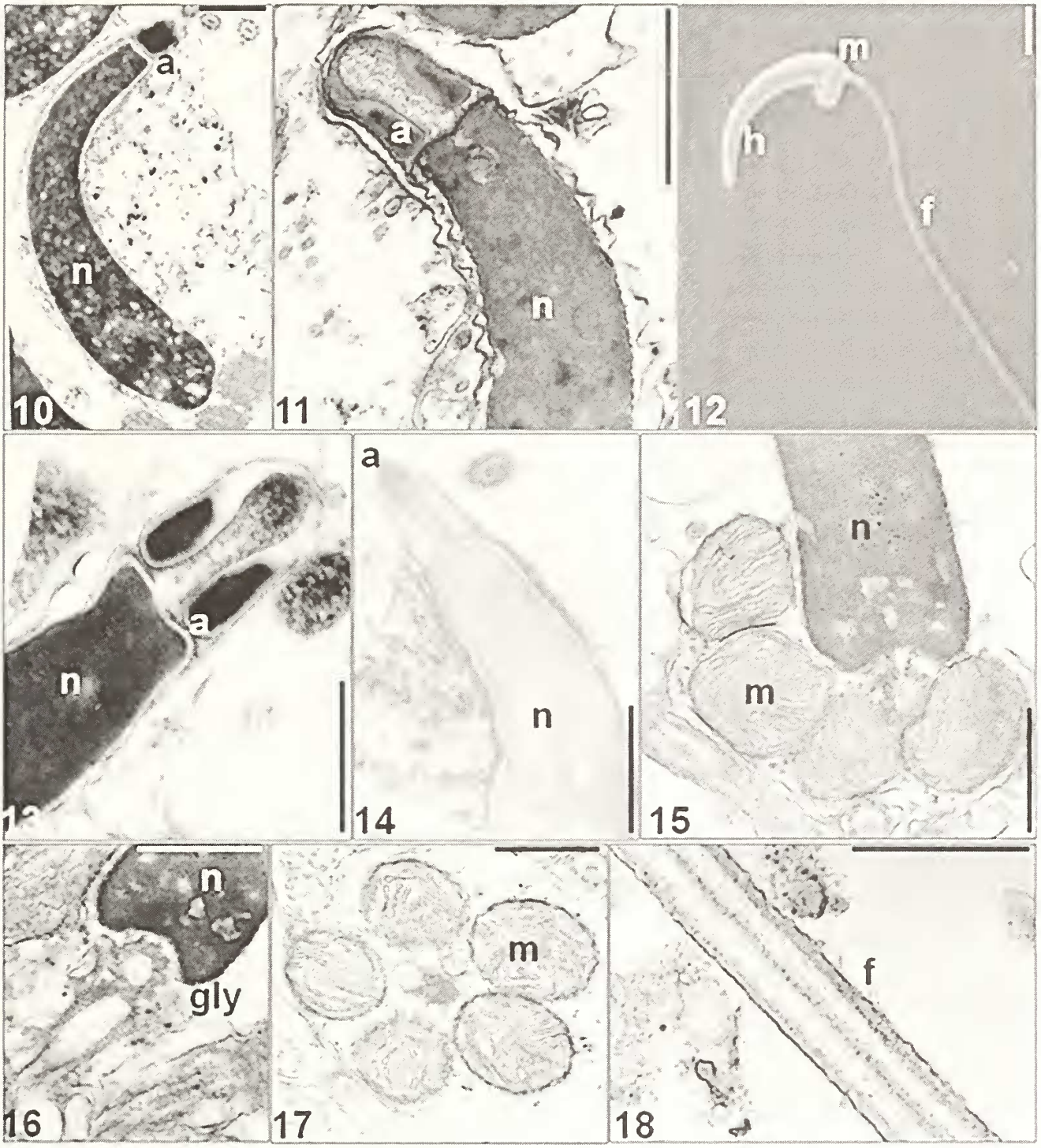
The morphological structures of the venerid spermatozoa described herein agree with the *ect-aquasperm* type proposed by Rouse and Jamieson (1987), exhibiting morphological characteristics described for free-spawning bivalves. In a previous study of bivalve sperm ultrastructure, Healy et al. (1995b) proposed five categories within the order Veneroida. The spermatozoa descriptions presented here are in agreement with the data reported by Healy et al. (1995b) regarding members belonging to Group A. Members of Group A share the following traits: a randomly organized subacrosomal material, an electron-lucent area at the acrosomal apex, a relatively long and slender rod nucleus that slightly decreases in thickness toward the gamete apex, and absence of an anterior nuclear fossa.

As described below, previous studies described detailed ultrastructural patterns of venerid spermatozoa; the reported data allows for an informal taxonomic analysis of this bivalve group.

Venerupis aurea (Gmelin, 1791) produces a spermatozoon with a very slightly curved nucleus and electron-dense regions at the base of the acrosomal vesicle (Gharagozlou and Pochon-Masson, 1971). Nicotra and Zappata (1991) investigated the sperm cells of *Callista chione* (Linnaeus, 1758), which also exhibit nuclear curvature and the same pattern of acrosomal electron density. Reunov and Hodgson (1994) described the spermatozoan morphology of the venerid clam *Tivela polita* (G.B. Sowerby II, 1851) from South Africa. The head of *T. polita* spermatozoa is about 3.2 µm long and has a cylindrical, slightly curved nucleus capped by a small conical acrosome. In the venerid clams *Protothaca thaca* (Molina, 1782) and *Ameghinomya antiqua* (King and Broderip, 1832) (as *Venus antiqua*), the reported length of the sperm head is about 7.5 µm and 5.3 µm, respectively, and the acrosome is a small vesicle in the anterior region of the cell (Guerra et al., 1994). Sperm ultrastructure studies of *Protothaca pectorina* (Lamarck, 1818) from the northern coast of Brazil showed that the male gamete exhibits a curved nucleus (Matos et al., 1997). The species *Gafrarium tumidum* Röding, 1798, and *Circe scripta* (Linnaeus, 1758) (Circinae), *Pitar sulfureum* Pilsbry, 1804 (Pitarinae), and *Gomphina aequilatera* (G.B. Sowerby I, 1825) (Tapetinae) share highly similar spermatozoa



Figures 1–9. Spermatozoon of *Anomalocardia brasiliana*. **1.** Acrosome, nucleus and midpiece. **2.** Aerosomal complex and nuclear apex. **3–4.** SEM showing head and flagellum. **5–6.** Phosphotungstic acid staining. **7.** Longitudinal section of the midpiece. **8.** Transversal section of the midpiece showing four spherical mitochondria grouped as a ring around the proximal centriole. **9.** Longitudinal section of the flagellum. Scale bars = 0.5 µm, except for Figure 3 = 10 µm and Figure 4 = 1 µm. Abbreviations: **a,** acrosome; **f,** flagellum; **h,** head; **m,** mitochondria; **n,** nucleus.



Figures 10–18. Spermatozoon of *Tivela mactroides*. **10.** Acrosome, nucleus and midpiece. **11.** Acrosomal complex and nuclear apex. **12.** SEM showing head and flagellum. **13.** Acrosomal complex and nuclear apex. **14.** Lack of phosphotungstic acid staining in the acrosomal vesicle. **15.** Longitudinal section of the midpiece, showing six spherical mitochondria grouped around the proximal centriole. **16.** Longitudinal section of the midpiece. **17.** Spherical mitochondria grouped around the distal centriole. **18.** Longitudinal section of the flagellum. Scale bars = 0.5 µm, except for Figure 12 = 1 µm. Abbreviations: **a,** acrosome; **f,** flagellum; **gly,** glycogen clusters; **h,** head; **m,** mitochondria; **n,** nucleus.

Table 1. Morphometric and numerical data of analyzed sperm structures in *A. brasiliana* and *T. mactroides*.

Species	Acrosomal length (μm)	Nuclear width (μm)	Head length (μm)	Number of mitochondria
A. brasiliana	0.4	0.9	3	4
T. mactroides	0.4	0.5	4.5	5–6

morphology and ultrastructure, as reported by Gwo et al. (2002). Sperm cells of these bivalve species consisted of a curved head, a short midpiece with tightly packed mitochondria and a long tail. The spermatozoa of the bivalve mollusks *Pitar rudis* (Poli, 1795) and *Chamelea gallina* (Linnaeus, 1758) (Veneridae) from Turkey were characterized by a conical and slightly curved nucleus about 2.6 μm and 3.5 μm long, respectively, and acrosomal vesicles about 0.6 μm long (Erkan and Sousa, 2002). The sperm cell of *Gomphina veneriformis* (Lamarck, 1818) exhibited a conspicuous curvature and the head (8.5 μm) is considered very long (Park et al., 2002). Sperm cells of the clam *Mercenaria mercenaria* (Linnaeus, 1758) (from China), were investigated by Ying et al. (2008), sharing common features with other venerid species, such as the presence of a curved nucleus and a short acrosomal vesicle which shows electron dense regions in its base.

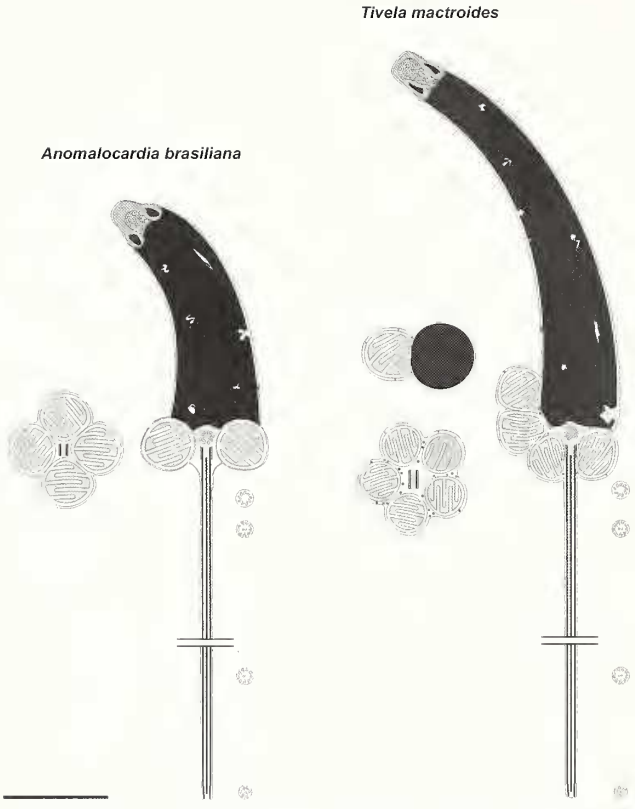


Figure 19. Diagrammatic representation of *A. brasiliana* and *T. mactroides* spermatozoa. Scale bar = 1 μm.

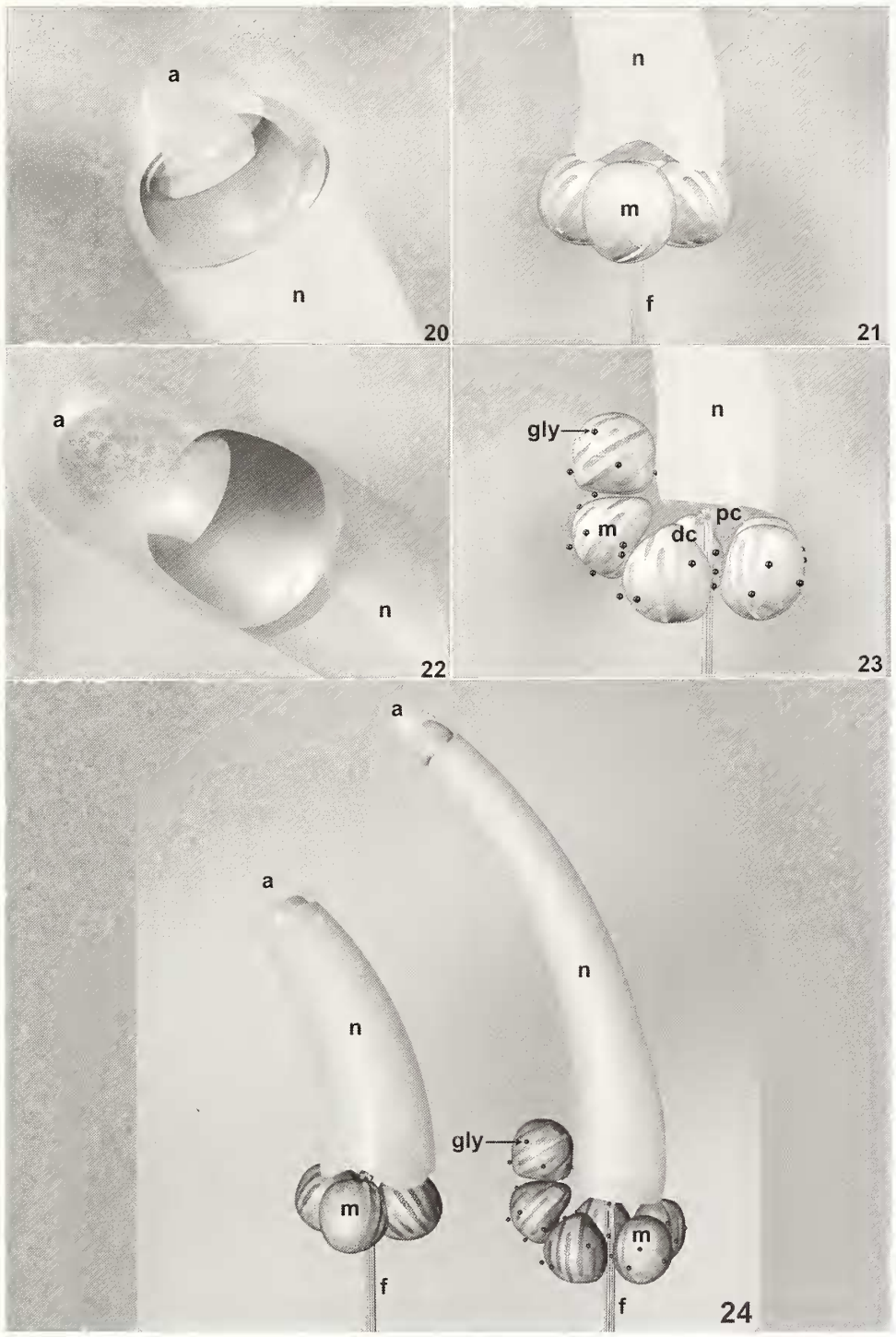
The elongated head of venerid spermatozoa usually contains a curved nucleus, which characterizes these bivalves (Reunov and Hodgson, 1994). However, the nucleus curvature is not unique to venerids since it has also been reported in galeommatoidean bivalves (Eckelbarger et al., 1990). Nicotra and Zappata (1991) stated that it should be interesting to analyze movement and fertilization patterns in this curved sperm, in order to evaluate the functional significance of this characteristic. However, considering the microscopic dimensions of the sperm cells, the nucleus shape should be irrelevant to the movement because these bodies have low Reynolds numbers, not being possible to derive any appreciable thrust from inertia.

The apical half of the spermatozoon acrosomal vesicle is stained with PTA in the species *Ruditapes decussatus* (Linnaeus, 1758) (as *Venerupis decussatus*), *Eurhomalea rufa* (Lamarck, 1818), *Protothaca thaca,* and *Venus antiqua* (family Veneridae), as reported by Sousa et al. (1998). This is in contrast to the negative PTA staining pattern of *Anomalocardia brasiliana* and *Tivela mactroides* spermatozoa.

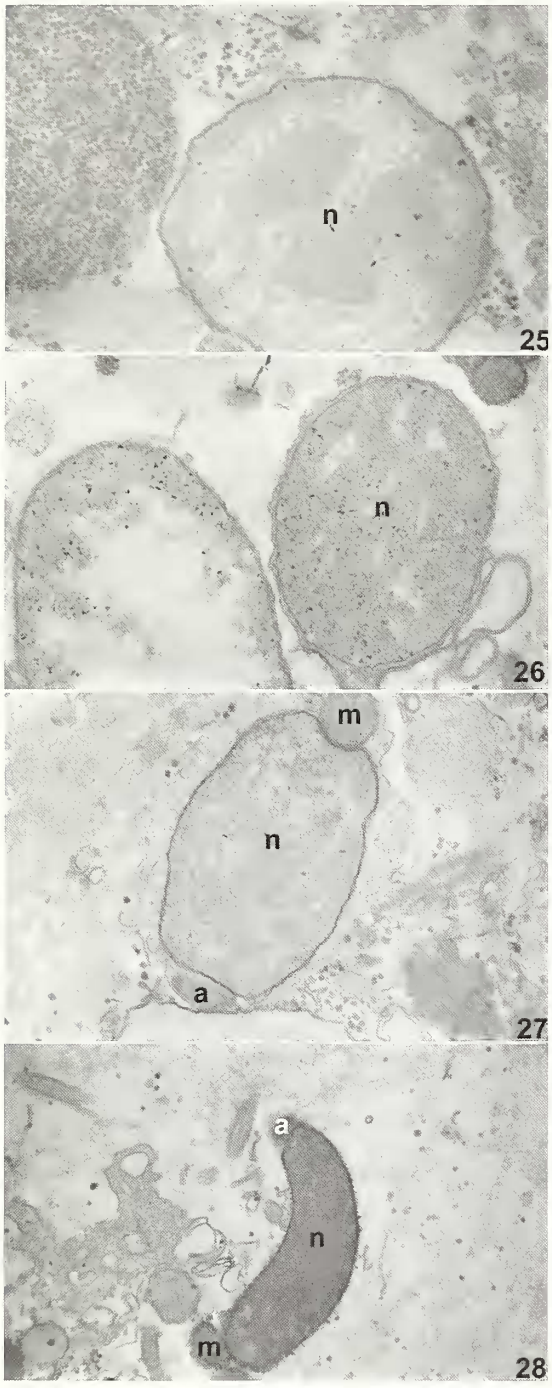
Sperm morphological studies described non-curved nucleus in the venerid *Eurhomalea rufa* (Sousa et al., 1998, Guerra et al., 2003). The nucleus of sperm cells were curved in all other studied venerid species. The ultrastructural characteristics of *Ruditapes decussatus* (Pochon-Masson and Gharagozlou, 1970; Gharagozlou and Pochon-Masson, 1971) were different in comparison to other venerid species, especially in relation to acrosomal features. It is important to emphasize that the sperm cell of *Eurhomalea rufa* shows significant morphological differences in comparison with other species of the family Veneridae. These sperm cells differences have not yet been explained, representing an invitation to a taxonomic review of these species.

Besides their similarities, *A. brasiliana* and *T. mactroides* spermatozoa showed prominent ultrastructural differences. The sperm nucleus was slightly curved in *A. brasiliana* and prominently curved and long in *T. mactroides*. In the *A. brasiliana* sperm cell midpiece, there were four mitochondria uniformly distributed around the centrioles. As for *T. mactroides*, there were six asymmetrically distributed mitochondria. Finally, the midpiece of *T. mactroides* contained glycogen deposits whereas that of *A. brasiliana* did not. These morphological patterns suggest that the *T. mactroides* spermatozoa could be adapted to turbulent environments.

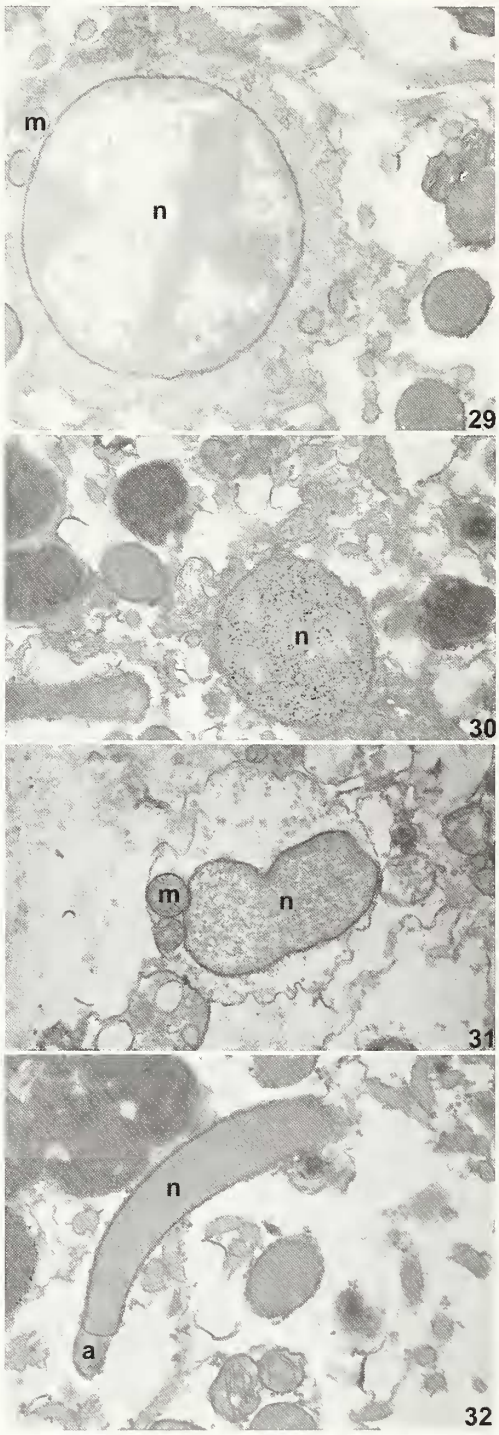
Narchi (1972) compared structural and functional morphologies as well as adaptations of *A. brasiliana* and *T. mactroides*, which are species living close to the surface in soft substrata with suspension-feeding habits. Their most prominent anatomical features were related to burrowing behavior and suspension-feeding. *Anomalocardia brasiliana*, which lives in calm waters of muddy beaches, does not have tentacles along the mantle edge whereas the inhalant siphon and mantle edge of *T. mactroides* have several ramified tentacles. The siphonal tentacles prevent the penetration of large particles into the mantle cavity while the ramified tentacles along the



Figures 20–24. Illustrations of spermatozoan features of *Anomalocardia brasiliana* and *Tivela mactroides* based on SEM and TEM images processed through 3D animation rendering and modeling software. **20.** Apex of the head of the spermatozoon of *A. brasiliana*. Acrosomal length = 0.4 µm. **21.** Middle piece of the spermatozoon of *A. brasiliana*. Nuclear width = 0.9 µm; mitochondrion = 0.55 µm. **22.** Apex of the head of the spermatozoon of *T. mactroides*. Acrosomal length = 0.5 µm. **23.** Middle piece of the spermatozoon of *T. mactroides*. Mitochondrion = 0.5 µm. **24.** General visualization of the spermatozoan features of both spermatozoa. Mitochondrion = 0.55 µm (in spermatozoan representation at right side of the figure.) Abbreviations: **a,** acrosome; **dc,** distal centriole; **f,** flagellum; **gly,** glycogen clusters; **h,** head; **m,** mitochondria; **n,** nucleus, **pc,** proximal centriole.



Figures 25–28. Precursory cells of *Anomalocardia brasiliana*. **25.** Early spermatids with spherical nucleus with patches of condensed chromatin in the middle and in the cell periphery. Note staining indicating the presence of nuclear ACPase. Nuclear diameter = 4.2 µm. **26.** Nucleus still rounded in mid-spermatid stage, but chromatin condensation was intensified (presence of nuclear ACPase). Nuclear width = 3 µm and nuclear length = 4 µm. **27.** Late spermatid characterized by elongation of nucleus (presence of nuclear ACPase). Mitochondrion = 0.6 µm. **28.** Nuclear ACPase not detected in the sperm cell. Mitochondrion = 0.6 µm. Abbreviations: **a,** acrosome; **m,** mitochondria; **n,** nucleus.



Figures 29–32. Precursory cells of *Tivela mactroides*. **29.** Early spermatids with spherical nucleus with patches of condensed chromatin in the middle and in the cell periphery. Mitochondrion = 0.6 µm. **30.** Nucleus was still rounded in mid-spermatid stage, but chromatin condensation was intensified (presence of nuclear ACPase). Nuclear diameter = 2.25 µm. **31.** Late spermatid characterized by the elongation of the nucleus. Mitochondrion = 0.6 µm. **32.** Nuclear ACPase not detected in the sperm cell. Mitochondrion = 0.6 µm. Abbreviations: **a,** acrosome; **m,** mitochondria; **n,** nucleus.

mantle edge prevent large particles from entering the pallial cavity. These adaptations allow *T. mactroidcs* to live on open sandy shores where large quantities of material are kept in suspension by constant wave movements. *Tivcla mactroidcs* also has the most efficient form of particle transport among demibranchs, which partly reflects adaptation to a specific habitat. In agreement with this characteristic, the stomach of *T. mactroidcs* is more complex than that of *A. brasiliana*, which is related to the large number of particles present in this organ. Our observations on the spermatozoan morphology of *A. brasiliana* and *T. mactroidcs* mostly agree with Narchi (1972), who concluded that anatomical variations in these species reflect adaptations to diverse environments. According to Anderson and Personne (1970; 1976) and Introíni et al. (2009), glycogen storage in the middle piece of bivalve sperm cells has an important meaning in the spermatozoon physiological metabolism. The presence of glycogen clusters in the mid-piece could extend the longevity of the sperm cells. Any increase in the life expectancy of sperm cells implies in an increase in the probability of finding eggs and increase in opportunities for fertilization. This could be an adaptive advantage in turbulent waters.

Spermiogenesis in Veneroida species has been described with great accuracy emphasizing architectural details of cells (Nicotra and Zapata, 1991; Johnson et al., 1996, Ying et al., 2008). In the present work, the relatively low fixation shown in the electron micrographs of precursory cells was carried out intentionally, in order to avoid masking detection of Nuclear ACPase activity.

Considerable ultrastructural modifications take place during spermiogenesis. The chromatin filaments aggregate into lamellar structures and finally into a homogeneous and compact DNA arrangement. In *Anomalocardia brasiliana* and *Tivcla mactroidcs* gonads, a nuclear acid phosphatase (ACPase) was detected in spermatids using the improved Gomori-chloride technique. In the present analysis, the nuclear acid phosphatase activity in both bivalve species follows a specific time and spatial-course pattern during spermatid chromatin condensation. A controlled and comparative study suggests that this pattern of nuclear acid phosphatase activity is specific and related to chromatin compaction.

In conclusion, our results suggest that detailed analyses of bivalve spermatozoan ultrastructure can be useful tools in the investigation of interspecific taxonomic relatedness and adaptation to a given environment. Further studies on male gametes of venerid mollusks are needed to verify the taxonomic relevance of sperm morphological and ultrastructural characteristics.

ACKNOWLEDGMENTS

The authors thank Dr. Alexander Turra and Dr. Márcia Denadai for helping with *Anomalocardia brasiliana* and *Tivcla mactroidcs* samplings. This work was supported by FAPESP (Grant no. 04/13887-4). M.S. was supported by POCI/SAU-MMO/60709/60555/59994/04; UMIB.

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